Exhibit A

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Cell Biology

Regulation of the polarization of T cells toward antigen-presenting cells by Ras-related GTPase CDC42

(cell polarity/cytoskeleton/actin/microtubules/cell–cell interactions)

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ABSTRACT **The mechanisms by which cells rapidly polarize in the direction of external signals are not understood. Helper T cells, when contacted by an antigen-presenting cell, polarize their cytoskeletons toward the antigen-presenting cell within minutes. Here we show that, in T cells, the mammalian Ras-related GTPase CDC42 (the homologue of yeast CDC42, a protein involved in budding polarity) can regulate the polarization of both actin and microtubules toward antigen-presenting cells but is not involved in other T-cell signaling processes such as those which culminate in interleukin 2 production. Although T-cell polarization appears dispensable for signaling leading to interleukin 2 production, polarization may direct lymphokine secretion towards the correct antigen-presenting cell in a crowded cellular environment. Inhibitor experiments suggest that phosphatidylinositol 3-kinase is required for cytoskeletal polarization but that calcineurin activity, known to be important for other aspects of signaling, is not. Apparent conservation of CDC42 function between yeast and T cells suggests that this GTPase is a general regulator of cytoskeletal polarity in many cell types.**

How cells polarize their contents in response to a gradient of external signal is not understood. In yeast, the *CDC42* gene is required for establishing an axis of polarity during vegetative division by budding (1, 2). *CDC42* encodes a Ras-related GTPase which assembles at the site of bud formation, toward which the cytoskeleton polarizes (2, 3). Yeast cells mutant for *CDC42* cannot form a bud because they cannot restrict cell surface growth to the bud site (1). Underlying this targeting defect, the actin cytoskeleton is not polarized. A human CDC42 protein, 81% identical to the yeast CDC42, is known (4, 5). The experiments presented in this paper investigate the role of human CDC42 GTPase in the cytoskeletal polarization of T cells toward antigen-presenting cells.

Polarization of helper T cells toward antigen-presenting cells, as mediated by the interaction of the T-cell antigen receptor with antigen bound to proteins encoded by the major histocompatibility complex, is an example of cell polarity that is rapidly induced in the direction of an external signal (6–8). Interaction between helper T cells and antigen-presenting cells is part of the fundamental regulation of the immune response. Upon contact between an antigen-presenting cell and a helper T cell, a tight interface forms between the two cells, and the T cell polarizes its cytoskeleton toward the antigen-presenting cell (6–8). Much is known about the signaling which occurs in the T cell in response to antigen presentation (9); however, how this signaling controls polarization of the cytoskeleton is not known.

METHODS AND MATERIALS

Cell Lines, Growth Medium, and Inhibitor Treatments. Murine 2B4 T-cell hybridomas and CH27 B-cell lymphomas were grown in standard medium [RPMI 1640/10% fetal bovine serum (HyClone)/50 μm 2-mercaptoethanol supplemented with penicillin, streptomycin, and glutamine; ref. 10]. Where inhibitors were used, T cells were pretreated for 20 min prior to mixing, and the inhibitor was present during incubation with antigen-presenting cells. As a control for nonspecific effects on viability, the antigen-presenting cells were pretreated with wortmannin; when this pretreatment was performed, T-cell polarization still occurred.

Constructs and Transfections. A two-step polymerase chain reaction was used to generate two point mutations in human *CDC42* (11). Mutagenic primers were G12V (Gly^{12}→Val mutation), 5′-ACCAACAGCCACATCGCCCA-3′, and D57Y (Asp^{57}→Tyr mutation), 5′-TCTTGGACTTTTTTATACTGCAGGG-3′. Flanking primers were 5′-CGGGATCCCCGGTGGAGAAGCTG-3′ (on the 5′ flank) and 5′-CGGAATTCGGCTCTGGAGAGATG-3′ (on the 3′ flank). Mutant and wild-type products were ligated into pcDNA3 (Invitrogen) via the *Bam*HI/*Eco*RI sites underlined. Sequence analysis confirmed the predicted mutations.

To generate stable transfectants, 10 μg of each construct and vector control were linearized with *Pvu* I and purified. Fragment and 10 μg of carrier DNA were added to 1×10^7 2B4 cells in Hanks' balanced salt solution (12). Mixtures were electroporated (13) and aliquoted into 96-well culture dishes to ensure independent origins of clones isolated. After 24 hr, G418 (1 mg/ml; GIBCO/BRL) was added to select for transfectant clones. Ten days later, ≈15 G418-resistant wells per plate were observed, suggesting that clones were derived from single cells. No G418-resistant clones were observed with carrier DNA alone. Twelve G418-resistant clones were saved for each allele in each of two separate transfections.

Stimulations, Immunofluorescence, and Scoring Methods. Conjugation/stimulation and immunofluorescence were performed essentially as described (7, 8). Twenty minutes after mixing of the T cells with antigen-presenting cells, samples were fixed (3.7% formaldehyde, 40 min, room temperature). The cells were washed (phosphate-buffered saline plus 0.1% bovine serum albumin, pH 6.5) and permeabilized (10 min, 0.1 mM Triton X-100). Microtubules were labeled with rat anti-tubulin monoclonal antibody (YOL1/34; Accurate Antibodies, Westbury, NY) followed by fluorescein-conjugated goat anti-rat IgG (Jackson ImmunoResearch). CH27 antigen-presenting cells were surface-labeled with fluorescein-conjugated goat anti-mouse IgG (Jackson ImmunoResearch); F-actin was labeled with 33 nM rhodamine-phalloidin (Molecular Probes) (14).

Measurement of Interleukin 2 (IL-2) Production. Stimulations and measurement of IL-2 production by transfectant clones were performed by standard methods (10, 15).

RESULTS

T-cell polarization can be studied with a T-cell hybridoma (2B4) and a B-cell lymphoma (CH27) presenting an appro-

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Abbreviations: IL-2, interleukin 2; PI, phosphatidylinositol.

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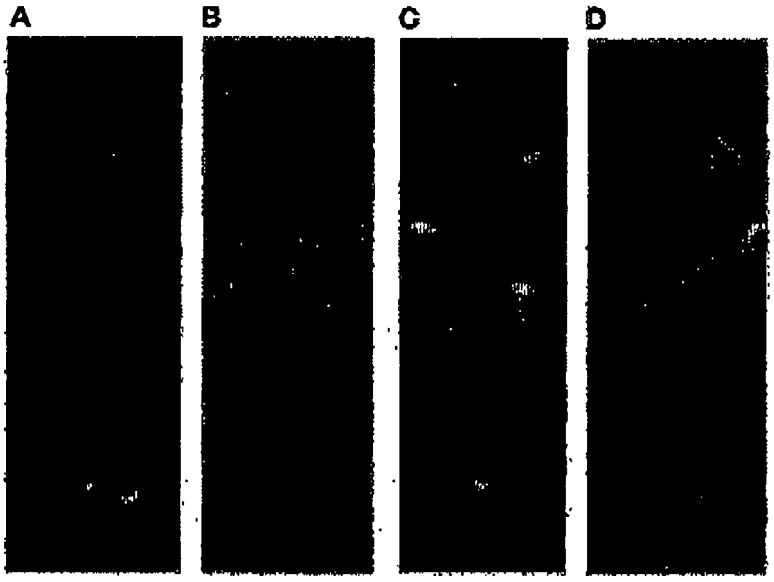

FIG. 1. Microtubule-organizing-center position in wild-type and mutant T cells coupled to antigen-presenting cells. (*A* and *B*) Untransfected T cells with microtubule-organizing centers polarized. (*C* and *D*) $CDC42^{G12V}$-transfected 2B4 T cells exhibiting a defect in microtubule-organizing-center polarization. *A* and *C* show indirect immunofluorescence of microtubules with antigen-presenting cells evident from cell surface labeling. *B* and *D* show differential interference contrast images of the same cells as in *A* and *C*. All panels are oriented with the T cell to the left. (Bar = 15 μm.)

priate antigen (6–8). Within 20 min of combining these two cell types, 90% of those T cells that have bound to antigen-presenting cells polarize their microtubule-organizing centers to be adjacent to the antigen-presenting cell (Fig. 1 *A* and *B*) and exhibit a concentration of polymerized actin beneath the plasma membrane at the site of contact (see Fig. 3 *B* and *C*).

To investigate the role of CDC42 in the polarized response of T cells, we examined the polarization properties of T cells transfected with two alleles of *CDC42* predicted to interfere with the functioning of the endogeneous *CDC42*. $CDC42^{G12V}$ is predicted to produce a CDC42 protein defective for GTP hydrolysis and, therefore, locked in the GTP-bound conformation (16, 17). $CDC42^{D57Y}$ is predicted to produce a CDC42 protein locked in a conformation mimicking the GDP-bound form; the same mutation in *RAS* produces a potent dominant-negative effect by sequestering its GDP–GTP exchange factor (18). Both alleles and wild-type *CDC42* (expressed from the constitutive cytomegalovirus promoter) were stably transfected into the murine 2B4 T-cell hybridoma. All three constructs and vector control yielded similar numbers of stable transfectant clones with indistinguishable growth rates (L.S. and D.Y., unpublished data). Thus, the expression of mutant *CDC42* alleles, in this context, did not interfere with cell growth or division. Transfected *CDC42* alleles were expressed at levels 2- to 5-fold above endogenous *CDC42*, as estimated by Western blot analysis, with some variation among clones (L.S., unpublished data).

Mutant and control T cells were mixed with antigen-presenting cells, and, after a 20-min incubation, the positions of the microtubule-organizing centers within the T cells were scored. All vector-alone and wild-type *CDC42* clones oriented their microtubule-organizing centers toward antigen-presenting cells with efficiencies similar to untransfected T cells (Fig. 1 *A* and *B*; Fig. 2*B*). In contrast, all $CDC42^{G12V}$ and $CDC42^{D57Y}$ clones examined

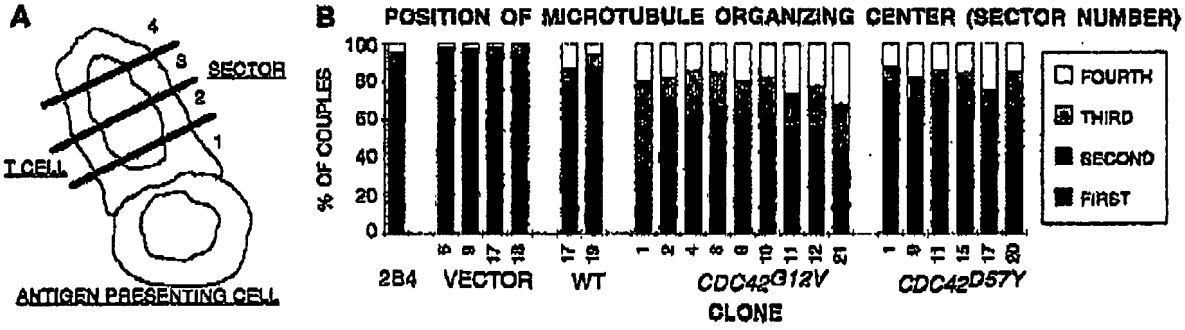

FIG. 2. Disruption of microtubule-organizing-center polarization in T cells expressing mutant alleles of *CDC42*. (*A*) Scoring the position of the microtubule-organizing center. The position was scored by dividing the T cell into four sections of equal width and assigning a 1, 2, 3, or 4 based on the location of the microtubule-organizing center relative to the antigen-presenting cell. In wild-type 2B4 cells, the microtubule-organizing center is predominantly in region 1 directly adjacent to the antigen-presenting cell—a polarized response. Only 2B4 cells that were coupled to a single CH27 cell were scored. (*B*) Summary of microtubule-organizing-center positions in wild-type (WT) and *CDC42*-mutant clones. Each lane corresponds to an independent clone. For each clone, two independent stimulations (≈100 couples scored for each stimulation) were averaged. For untransfected 2B4 cells, 1036 couples were scored.

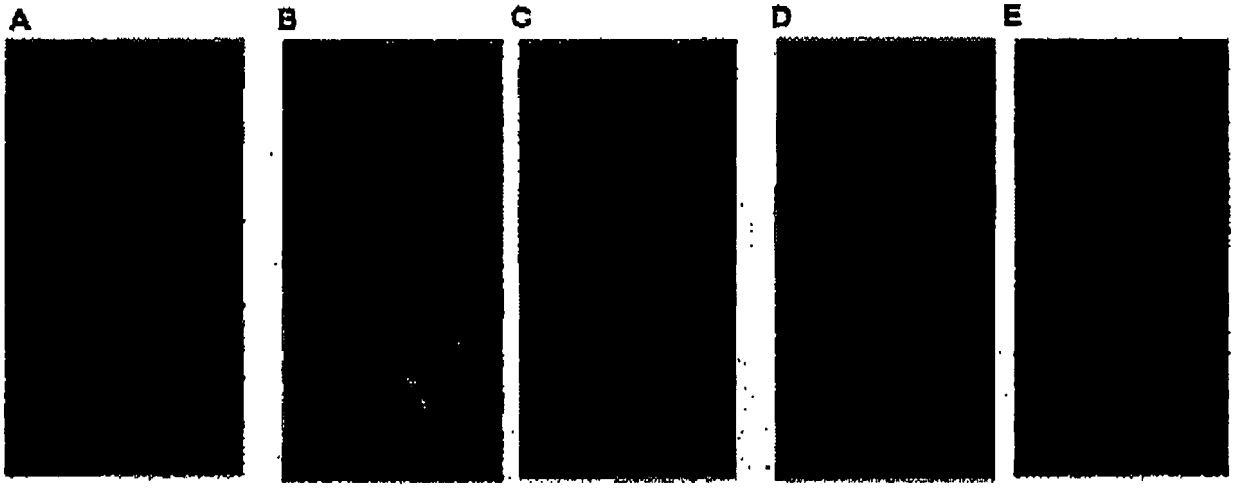

FIG. 3. Effects of mutant *CDC42* alleles on actin organization. (*A*) Examples of actin localization in unstimulated 2B4 T cells. This resting distribution was not altered in cells expressing either mutant allele of *CDC42*. (*Upper*) Focal plane illustrating the network of membrane-associated actin. (*Lower*) Focal plane illustrating the aggregate of cytoplasmic actin. (*B*) Examples of filamentous actin at the interface between the T cell and the antigen-presenting cell. (*D*) Lack of actin reorganization in $CDC42^{D57Y}$-expressing cells upon encountering an antigen-presenting cell ($CDC42^{G12V}$-expressing cells are identical in appearance). (*C* and *D*) Antigen-presenting cells identified by cell surface labeling.

were defective in polarizing their microtubule-organizing centers toward antigen-presenting cells. The $CDC42^{G12V}$ clones exhibited randomly positioned microtubule-organizing centers when coupled to antigen-presenting cells (Fig. 1 *C* and *D*; Fig. 2 *A* and *B*). The $CDC42^{D57Y}$ clones exhibited a similar, but slightly less severe, defect (Fig. 2*B*).

Actin polarization was also disrupted in the *CDC42* mutant cell transfectants. In unstimulated T cells, mutant *CDC42* had no effect upon the actin distribution, which appeared as a cortical meshwork and a cytoplasmic concentration in the vicinity of the microtubule-organizing center (Fig. 3*A*). However, the actin distribution in stimulated cells was affected by the *CDC42* mutant alleles. In 90% of wild-type T cells coupled to antigen-presenting cells, polymerized actin accumulated at the interface with the antigen-presenting cell (Fig. 3 *B* and *C*). Expression of either mutant allele greatly reduced the number of T cells exhibiting polarized actin (Table 1); most showed no polarized actin assembly (Fig. 3 *D* and *E*). The number of mutant cells showing actin polarization (15–20%) is likely to be an overestimate because actin polarization in the antigen-presenting cell sometimes produced an interfering fluorescence signal; when unclear, T cells were scored as polarized.

To test the effects of preventing polarization upon signaling within the T cell, IL-2 production by T-cell transfectants was measured (D.Y., unpublished data). All stable clones (except one $CDC42^{G12V}$ and one $CDC42^{D57Y}$ clone; not included in data presented) produced IL-2 at levels comparable to control cells in response to antigen-presenting cells. None of the clones produced detectable IL-2 in the absence of antigen presentation. Thus, signaling leading to transcription of the IL-2 gene occurred in the apparent absence of cytoskeletal polarization.

We therefore examined whether molecules known to be required for signaling to the nucleus were required for polarization. As presented in Fig. 4 and Table 1, inhibitors of calcineurin (FK506; ref. 19) and of the S6 kinase pathway (rapamycin; refs 20–22) had no effect on T-cell polarization. Wortmannin, an inhibitor of phosphatidylinositol (PI) 3-kinase (23), abolished cytoskeletal polarization. Thus, calcineurin and the S6 kinase pathway are dispensable for cytoskeletal polarization, but PI 3-kinase appears to be required.

Table 1. Effects of *CDC42* alleles and signaling inhibitors on actin polarization in 2B4 cells

Transformation or treatment	% of cell couples	
	Polarized	Diffuse
CDC42 allele		
None	86	14
Vector	94	6
$CDC42^{G12V}$	15	85
$CDC42^{D57Y}$	21	79
Cell treatment		
Dimethyl sulfoxide	92	8
Wortmannin (100 nM)	0	100
FK506 (100 nM)	91	9
Rapamycin (100 nM)	93	7

Coupled T cells were scored as to whether an accumulation of actin was visible at the interface with the antigen-presenting cell. Several clones of each mutant allele were scored and data were averaged.

DISCUSSION

The data demonstrate that both constitutively active and dominant negative alleles of *CDC42* prevent cytoskeletal polarization of T cells. These effects can be explained by the hypothesis that, in wild-type T cells, CDC42 is converted to its GTP-bound form specifically at the site of contact with the antigen-presenting cell (Fig. 5*A*)—a situation that is prevented by expression of either mutant *CDC42* allele (Fig. 5 *B* and *C*). In wild-type T cells, CDC42 activation at the interface could occur if a GDP–GTP exchange activity within the T cell were concentrated in this region in response to a transmembrane receptor such as the T-cell antigen receptor. Spatially restricted, active GTP-bound CDC42 would then promote cytoskeletal assembly at the interface via the recruitment of microtubule-capture and actin-nucleation sites.

The effects of the different *CDC42* mutant alleles can be explained as follows: $CDC42^{G12V}$ (predicted to be constitutively GTP-bound; ref. 16; Fig. 5*B*) may provide a constitutive signal that masks the activation of endogenous CDC42, resulting in a polarization defect. $CDC42^{D57Y}$ most likely sequesters its guanine nucleotide exchange factor so that it cannot activate endogenous CDC42 in response to cell–cell signaling (ref. 18; Fig. 5*C*). $CDC42^{D57Y}$-expressing cells may show a less severe defect than the $CDC42^{G12V}$-expressing cells because sequestration of the exchange factor is not complete,

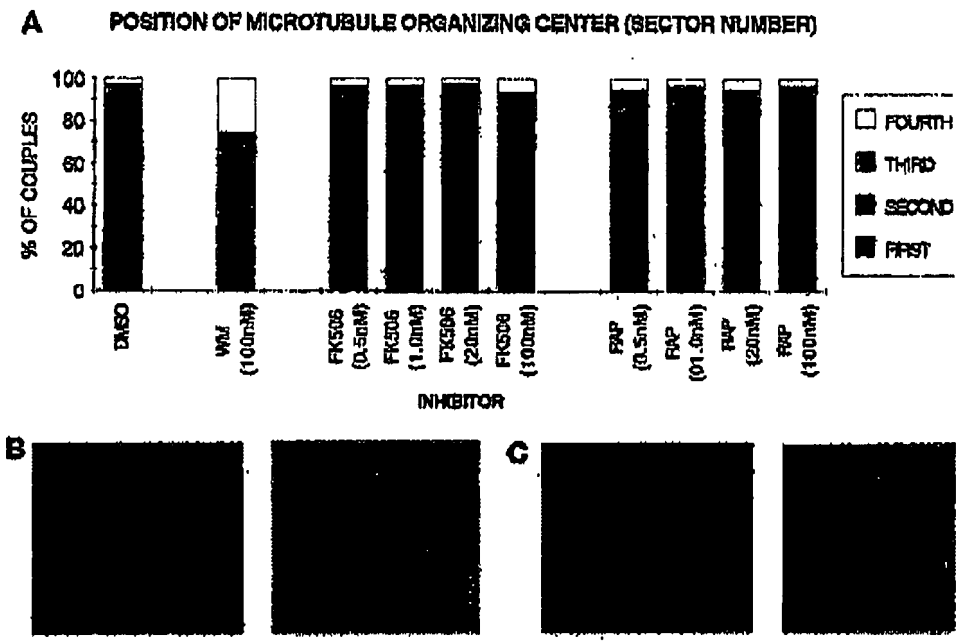

FIG. 4. Effects of inhibitors of signaling on cytoskeletal polarization. (*A*) Effects of wortmannin (WM), FK506, and rapamycin (RAP) on microtubule-organizing-center position. DMSO, dimethyl sulfoxide (solvent for delivery of inhibitors). (*B*) Microtubule-organizing-center position in wortmannin-treated T cells coupled to antigen-presenting cells. (*Left*) Microtubules. Surface labeling of the antigen-presenting cell. (*Right*) Differential interference contrast image of the same cells. (*C*) Actin distribution in wortmannin-treated T cells coupled to antigen-presenting cells. (*Left*) Filamentous actin. (*Right*) Surface labeling of the antigen-presenting cell. Panels in *B* and *C* are oriented with the T cells to the left.

allowing some endogenous CDC42 to be activated at the interface with the antigen-presenting cell.

During the course of these experiments, an important consideration has been whether the effects of the dominant alleles of *CDC42* reflect the normal function of CDC42 or are due to interference with some other pathway, such as those governed by other GTPases such as Ras, Rho, or Rac. Several lines of evidence, enumerated below, strongly argue that the effects of the mutant alleles are specific and reflect the normal role of CDC42. (*i*) Expression of the alleles of *CDC42* had no effect on growth rate, viability, or coupling efficiency between T cells and antigen-presenting cells; therefore, expression of the mutant alleles of *CDC42* did not interfere with basic T-cell function. (*ii*) Signaling, such as that which culminates in IL-2 production and which involves Ras GTPase, appeared normal in the *CDC42* mutant cells. (*iii*) The mutant alleles of *CDC42* were overexpressed to a modest extent (2- to 5-fold). Considering the affinity of proteins for their normal targets, rather than heterologous targets, we think it unlikely that modest overexpression inappropriately affects other pathways. (*iv*) The observation that two alleles, which interfere by different mechanisms, specifically prevent the same process argues strongly that CDC42 is normally involved in this process. The possibility that two mechanistically different alleles inappropriately affect the same heterologous pathway is remote. (*v*) The effects of mutant alleles of *CDC42* are qualitatively distinct from those reported for similar alleles of the related GTPases Rac and Rho (24, 25). (*vi*) Finally, expression of these mutant alleles of mammalian *CDC42* in yeast produced the predicted phenotypes for related alleles of yeast *CDC42*, but not the phenotypes of *RHO* mutants of yeast (L.S., unpublished data). Although the possibility that the effects seen for expression of dominant alleles of *CDC42* are nonspecific cannot be eliminated with certainty, the sum of the evidence presented strongly supports the conclusion that CDC42 is normally involved in regulating the orientation of axes of polarity in T cells.

How does signaling control the polarization of the cytoskeleton? The observation that *CDC42* mutant cells defective for polarization are able to produce IL-2 suggests that CDC42 and cytoskeletal polarization may be controlled by a specialized pathway of signaling. Evidently, this pathway does not include calcineurin, which modulates signaling resulting in activation of the IL-2 gene (19), or the S6 kinase pathway, which modulates responses of the T cell to IL-2 (20–22). PI 3-kinase, however, is required for polarization of the cytoskeleton. PI 3-kinase is one of several lipid kinases identified, which are potentially involved in signal transduction, but whose exact role is unclear. PI 3-kinase is composed of two subunits: p85, a regulatory subunit, and p110, a catalytic subunit. The

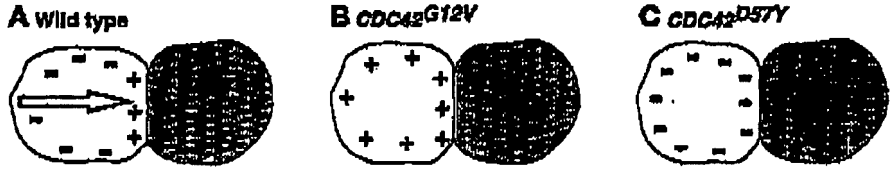

FIG. 5. A hypothesis to explain how CDC42 governs the orientation of cell polarity. See text for discussion. (*A*) Wild-type T cells. (*B*) $CDC42^{G12V}$-expressing cells. (*C*) $CDC42^{D57Y}$-expressing cells. +, GTP-bound CDC42; −, GDP-bound CDC42; nonshaded cell, T cell; shaded cell, antigen-presenting cell; arrow, axis of polarity.

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requirement for PI 3-kinase is particularly interesting since CDC42 has been reported to interact with p85 through a domain with similarity to GTPase-activating proteins (26).

The observation that polarization of the T cell is dispensable for signaling to the nucleus and activation of transcription of the IL-2 gene raises the issue of the role of cytoskeletal polarization in communication between the T cell and antigen-presenting cell. Although it is possible that the behavior of this T-cell hybridoma does not reflect the behavior of T cells *in vivo*, it is worth considering the possibility that polarization of the T cell, instead of being involved in receiving a signal, is required for sending signals in the correct direction; i.e. polarization may restrict the delivery of secreted lymphokines to the appropriate antigen-presenting cell, but not nearby cells (8). Ensuring that communication occurs only between the T cell and the appropriate antigen-presenting cell is likely to be important for maintaining the specificity of the immune response.

Our experiments support the conclusion that CDC42 is involved in polarizing the cytoskeletons of the T cells in the direction of an external signal, the antigen-presenting cell. Previous work has shown that the related GTPases Rho and Rac govern the structure of polymerized actin (stress fibers or ruffles; refs. 24 and 25), but not the overall orientation of the actin cytoskeleton. In contrast, expression of mutant *CDC42* prevented redistribution of actin from a cortical meshwork to a tight band at the T cell/antigen-presenting cell interface. Our observations also show that CDC42 affects the orientation of the microtubule cytoskeleton. To date, the related GTPases Rho, Rac, and yeast CDC42 have been reported to affect only actin organization. Functional conservation of CDC42 between yeast and mammals, as well as the expression of *CDC42* in all tissues examined to date (4, 5), suggests that CDC42 may govern a wide variety of polarized cellular behaviors such as axonal outgrowth (27), polarization of epithelial cells, and cell migrations.

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